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Role of actin dynamics in the cooperative maintenance of synaptic plasticity

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ABSTRACT

Activity-dependent changes in synaptic efficacy such as long-term potentiation (LTP) are widely accepted to be cellular mechanisms underlying memory and learning processes. Upon induction, LTP can be expressed as a transient or as a maintained form, depending on the stimulation strength. According to the Synaptic Tagging and Capture hypothesis (STC), the maintenance of activity-dependent changes are depended on the interplay between an input-specific synaptic tag and the capture of plasticity-related proteins (PRPs). Given that maintained forms of LTP depend on PRPs capture, the activated synapses can undergo either synaptic cooperation or competition, depending on proteins availability.

Actin cytoskeleton dynamics has been suggested as a major candidate for the synaptic tag role. Actin plays a critical role in synaptic plasticity, linking functional and structural plasticity. Induction of LTP leads to activation of CaMKII, which in turn leads to activation of several small GTPases such as Cdc42, a molecule involved in actin cytoskeleton remodeling during plasticity induction. Cdc42 modulates actin network in an activity-dependent manner and once activated Cdc42 is spatially restricted to the stimulated spine. Actin network assembling and disassembling leads to structural changes on dendritic spines and pharmacological modulation of the actin dynamics affects LTP maintenance, suggesting that the remodeling of the actin provides the molecular signal (synaptic tag) for PRPs capture.

Here, we aim to assess the molecular mechanisms underlying the setting for the synaptic tag. To do this, we use a specific inhibitor of Cdc42 activation (ML141) and we investigate the role of Cdc42 activation in the synaptic tag modulation during LTP induction and expression, and also during synaptic cooperation and competition. Our results showed that inhibition of Cdc42 for 30 min does not interfere with the induction of transient forms of LTP, while the induction of persistent forms of LTP are Cdc42 activation-dependent. Moreover, our results showed that maintained forms of LTP can be destabilized if Cdc42 inhibition occurs within a specific time-window. Inhibition of Cdc42 70 min upon LTP induction does not interfere with LTP maintenance. We also test the role of Cdc42 activation in both synaptic cooperation and competition. We showed that inhibition of Cdc42 blocks the ability of synapses to cooperate, however the synaptic capture impairment induced by ML141 application was reverted when an actin polymerization inhibitor (cytochalasin) was added or when activity was suspended. In synaptic competition, we showed that inhibition of Cdc42 leads to the maintenance of all stimulated inputs.

Our data showed that activation of Cdc42 is a key regulator of the synaptic tag modulation. We showed that inhibition of the Cdc42 clearly compromises LTP induction and maintenance, and both synaptic cooperation and competition were also affected. Together, our data provide strong evidence that supports the actin cytoskeleton as a potential synaptic tag.

KEYWORDS: Synaptic plasticity; Actin cytoskeleton; STC; Synaptic tag; Cdc42

RESUMO

As formas de plasticidade sináptica dependentes de atividade, tal como a potenciação de longa duração (LTP, do inglês *long-term potentiation*) e a depressão de longa duração (LTD, do inglês *long-term depression*) são mecanismos celulares associados aos processos de memória e aprendizagem. A LTP é a forma de plasticidade sináptica dependente de atividade mais estudada. O mecanismo molecular envolvido na indução de LTP é complexo, e envolve a ativação de diversas vias de sinalização. A indução é iniciada com a entrada de iões de cálcio para os neurónios pós-sinápticos através dos recetores de glutamato N-Metil-D-Aspartato (do inglês NMDA, *N-methyl-D-aspartate*). A entrada de cálcio através deste recetor conduz a ativação da proteína quinase II dependente de cálcio e calmodulina (do inglês CaMKII, Calcium /Calmodulin-dependent protein kinase II). Uma vez ativada, esta proteína conduz a translocação dos recetores alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico (do inglês AMPA, *α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)* para a sinapse, potencializando a transmissão sináptica. Este mecanismo está geralmente associado à indução de uma forma de LTP transiente. Para que as alterações na eficácia sejam mantidas, estas necessitam de mecanismos de transcrição e tradução de modo a ocorrer a síntese de proteínas associadas a fenómenos de plasticidade (PRPs-do inglês *plasticty-related proteins*) e de um marcador sináptico que irá capturar estas proteínas. Esta hipótese foi sugerida pela primeira fez em 1997 pelos investigadores Uwe Frey e Richard Morris, sendo classificada de hipótese da marcação e captura sináptica (do inglês STC, *Synaptic Tagging and Capture hypothesis*). Dado que as PRPs são apenas sintetizadas em resultado de uma tetanização forte, enquanto que a exibição do marcador pelas sinapses ocorre em ambas as tetanizações (fraca ou forte), fenómenos de cooperação e competição sináptica podem ocorrer no contexto da hipótese de STC. A cooperação sináptica define-se pela estabilização das formas transientes de LTP através da partilha de PRPs entre sinapses. As sinapses estimuladas com uma tetanização fraca podem cooperar com as sinapses estimuladas com uma tetanização forte permitindo deste modo a sua estabilização através da partilha de PRPs disponíveis. Por outro lado, a competição sináptica ocorre quando existe uma menor disponibilidade de proteínas ou maior número de sinapses ativadas, e por consequência mais marcadores disponíveis para a capture de PRPs.

Apesar de a hipótese do STC ter sido descrita há mais de 20 anos, a identidade molecular do marcador sináptico continua a ser alvo de intensa investigação. O citoesqueleto de actina tem sido colocado com um potencial candidato para o papel de marcador sináptico. O citoesqueleto de actina apresenta um papel importante quer na plasticidade funcional como na plasticidade estrutural. Estudos anteriores sugerem que a modulação da rede de actina, através de fármacos que afetam a sua polimerização ou despolimerização, interfere com as formas persistentes da LTP. Por outro lado, estudos revelam que a modulação da dinâmica da actina conduz a alterações estruturais nas espinhas dendríticas.

A dinâmica da actina envolve uma via de sinalização complexa em várias proteínas que se podem ligar a ela, chamadas de proteínas que se ligam à actina (do inglês ABP, Actin-binding proteins). Uma destas proteínas é CaMKII cuja sua ativação conduz à modulação de diversas moléculas a jusante, tais como Cdc42, um membro da família das GTPases. Esta molécula tem como principais caraterísticas a sua capacidade de modelar o citoesqueleto actina de uma forma dependente da atividade e o facto de esta ser espacialmente limitada às espinhas dendríticas estimuladas. Dadas as características da Cdc42, nós propomos que esta molécula representa um papel importante na modulação do *setting* do marcador sináptico.

Neste trabalho, pretende-se investigar o papel da Cdc42 na plasticidade sináptica, utilizando um inibidor seletivo da mesma (ML141). Nós estudámos a importância da ativação desta molécula na indução e expressão de formas transientes e persistentes de LTP, bem como o seu papel nos fenómenos de cooperação e competição sináptica. De modo a investigar as questões acima mencionadas, os

potenciais excitatórios pós-sinápticos desencadeados pela estimulação dos colaterais de Schaffer foram registados no *stratum radiatum* da área CA1.

Os nossos resultados demostram que a indução de formas transientes de LTP não são afetadas pela inibição da Cdc42. No entanto, a indução de formas persistentes de LTP requerem a ativação da Cdc42. Por outro lado, nós demonstramos que a ativação da Cdc42 é também necessária para a manutenção da plasticidade, no entanto a sua ativação apenas é necessária dentro de uma janela temporal especifica. Os nossos resultados demostraram que as formas persistentes de LTP podem ser destabilizadas se a inibição da Cdc42 ocorrer até 70 min após a indução de LTP.

Nós também avaliamos o efeito da inibição da Cdc42 na cooperação e competição sináptica. Os nossos dados revelam que a inibição da Cdc42 conduz ao bloqueio da cooperação sináptica. As formas transientes de LTP não são capazes de cooperar com a formas persistentes de LTP, uma vez que a inibição da Cdc42 leva a destabilização do marcador sináptico, conduzindo deste modo a uma captura ineficiente de PRPs. No entanto, o bloqueio da cooperação sinápica pode ser revertido se a aplicação de ML141 for simultânea com a da citocalasina (um inibidor da polimerização da actina) ou se a aplicação de ML141 for simultânea com a suspensão da atividade sináptica. Na competição sináptica, os nossos resultados demostraram que a inibição de Cdc42 leva a estabilização de todos os *inputs* ativados.

Em suma, os nossos resultados fornecem fortes evidências de que a Cdc42 desempenha um papel importante na hipótese do STC. Os resultados obtidos demostraram que a inibição do Cdc42 compromete a indução e a manutenção da LTP, assim como interfere com os mecanismos de cooperação e competição, através da modulação do citoesqueleto de actina.

PALAVRAS CHAVE: Plasticidade sináptica, citoesqueleto de actina, STC, marcador sináptico, Cdc42

INDEX

ABBREVIATIONS

ABP Actin binding proteins

ACSF Artificial cerebrospinal fluid

ADP Adenosine diphosphate

AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ATP Adenosine triphosphate

BDNF Brain-derived neurotrophic factor

Ca^{2+} Calcium ions

CaMKII Calcium/ calmodulin-dependent kinase II

Cdc42 Cell division control protein 42 homolog

DMSO Dimethylsulfoxide

EPSPs Excitatory-postsynaptic potentials

GEFs Guanine nucleotide exchange factor

GDIs Guanine nucleotide dissociation inhibitors

GTP Guanosine triphosphate

HFS High-frequency stimulation

LIMK LIM kinase

LTD Long-term depression

LTP Long-term potentiation

NMDA N-methyl-D-aspartate

N-WASP neuronal Wiskott-Aldrich syndrome protein

PAK serine/threonine kinase p21-activated kinase

PPF Paired-pulse facilitation

PRPs Plasticity-related proteins

PSD postsynaptic density

Rac1 Ras-related C3 botulinum toxin substrate 1

RhoA Ras homolog gene family, member

STC Synaptic tag and capture

SHH Slingshot

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1. INTRODUCTION

1.1 Synaptic Plasticity: Mechanism and Function

Brain plasticity, also known as neuroplasticity, can be defined as the ability of the individuals to adapt to an evolving environment. The capacity of the neurons and neuronal circuits to continuously change in both structure and function, in response to environmental demands, is critical for brain development and also for memory and learning processes (Kolb & Whishaw, 1998; Markham & Greenough, 2004; Sale, Berardi, & Maffei, 2014). The major mechanism by which the neurons and neural circuits are changed is via modification of the synaptic transmission. The synapses have the ability to change the strength or the efficacy of the its transmission in an activity-dependent manner, a mechanism called synaptic plasticity (Abbott & Nelson, 2000; Ho, Lee, & Martin, 2011).

The synaptic plasticity phenomenon present bi-directional changes, such as long-term potentiation (LTP) and long-term depression (LTD) (Bear & Malenka, 1994). Depending on the pattern of stimulation, synapses can increase or decrease the strength of their transmission: LTP can be defined as a long-lasting activity-dependent enhancement of excitatory synaptic efficacy, while LTD is a long-lasting decrease in synaptic efficacy (Citri & Malenka, 2008; Kumar & Cunha, 2011). Induction of synaptic plasticity forms (LTP and LTD) involves changes at both structural and functional levels (Markham & Greenough, 2004; S. J. Martin, Grimwood, & Morris, 2000). The functional synaptic plasticity represents the activity-dependent modifications of synaptic transmission strength that includes the trafficking of receptors to or from the postsynaptic compartment and the modulation of the neurotransmitter release (Herring & Nicoll, 2016; Leenders & Sheng, 2005). The structural synaptic plasticity represents the activity-dependent changes that occur at dendritic spines, contributing to the consolidation of the functional changes. Dendritic spines were first described by Ramon y Cajal in 1888 and represent small protrusions arising from the dendrites, receiving most of the excitatory inputs of the brain (Nimchinsky, Sabatini, & Svoboda, 2002). In response to neuronal activation, dendritic spines can enlarge during LTP and shrink during LTD induction (Bosch & Hayashi, 2012; Citri & Malenka, 2008; Nägerl, Eberhorn, Cambridge, & Bonhoeffer, 2004; Zhou, Homma, & Poo, 2004).

LTP is the most studied form of synaptic plasticity and also one of the most attractive cellular models for memory acquisition. Several studies showed that LTP and memory acquisition share common properties such as cooperativity, associativity and input-specificity (Bliss & Collingridge, 1993; Bliss & Lomo, 1973; Whitlock, Heynen, Shuler, & Bear, 2006). LTP is cooperative because the postsynaptic depolarization needed to induce LTP is achieved by the activation of multiples inputs at the same time. The associativity feature of LTP is observed when a weak input is paired with a strong independent input, leading to LTP expression in the weakly stimulated input (Kitajima & Hara, 1991). Finally, LTP is input-specific due to its expression only at the stimulated synapses (Bliss & Cooke, 2011; Nicoll, 2017).

LTP was described for the first time on the dentate gyrus of the hippocampus by Bliss and Lomo in 1973, and since its discovery, LTP was also demonstrated in different brain regions (including the cerebral cortex, amygdala, and cerebellum) and several animal models (rodents, primates) (Bliss & Lomo, 1973; Citri & Malenka, 2008; Park et al., 2014; Zorumski & Izumi, 2013). The best-characterized type of LTP is the one induced by tetanic stimulation on the Shaffer collaterals CA3-CA1 hippocampal area, a well-known N-methyl-D-aspartate (NMDA) receptor-dependent LTP (Baltaci, Mogulkoc, & Baltaci, 2019; Lüscher & Malenka, 2012). The molecular mechanisms involved in LTP induction are complex and involve the activation of several signaling pathways. Typically, LTP can be divided into at least two stages: a transient form of LTP (also known as early-LTP, E-LTP) and a maintained form of LTP (also known as late-LTP, L-LTP). The induction of LTP is initiated with the entry of calcium

ions (Ca^{2+}) into postsynaptic neurons through the activated NMDA receptors (Gipson & Olive, 2016; Grover & Teyler, 1998). The calcium influx through the NMDA receptor is considered to be the key signal to trigger LTP, leading to activation of Ca2+/calmodulin-dependent protein kinase (CaMKII), a key regulatory enzyme of this plasticity. Upon activation of CaMKII, specific proteins are translocated to the postsynaptic membrane including AMPA-type glutamate receptors (AMPAr), leading to the potentiation of synaptic transmission (Hayashi et al., 2016; Herring & Nicoll, 2016; Lu et al., 2001). This mechanism leads to a transient form of LTP that decays to the baseline values after a few hours (Uwe Frey, Huang, & Kandel, 1993).

Previous studies on hippocampal LTP have suggested that gene expression and the *novo* protein synthesis are required for the expression of a maintained form of LTP. The evidence supporting this idea comes from previous studies where it was shown that application of protein synthesis inhibitors, such as anisomycin, are able to block the stabilization of the late phase of LTP (Uwe Frey, Krug, Reymann, & Matthies, 1988; Kelleher, Govindarajan, & Tonegawa, 2004; Krug, Lössner, & Ott, 1984). The induction of protein synthesis and consequently the induction of a maintained form of LTP requires a strong tetanic stimulation (Uwe Frey & Morris, 1998; Nguyen & Kandel, 1997).

Taking into consideration the input-specificity of the LTP, how the newly synthesized proteins (also known as plasticity-related proteins-PRPs) identify the stimulated synapse? The synaptic tagging and capture hypothesis (STC) proposed a mechanism about how these PRPs interact only with the activated synapses.

1.2 Synaptic Tagging and Capture Hypothesis

The STC model was proposed by Frey and Morris in 1997 and it seeks to explain how the input specificity of LTP and its dependence on protein synthesis are conciliated during induction of plasticity. This hypothesis states that the stimulated synapses exhibit a "synaptic tag" which allows them to capture the newly synthesized PRPs in an input-specific manner, leading to the stabilization of the synaptic changes. The authors showed that induction of a long-lasting form of LTP (strong stimulation) in one set of Shaffer collaterals is able to stabilize the transient form of LTP (weak stimulation) induced in a second independent set of Shaffer collaterals (U. Frey & Morris, 1998; Uwe Frey & Morris, 1997; K. C. Martin & Kosik, 2002; Reymann & Frey, 2007). Consisting with this, the STC hypothesis proposed that long-lasting maintenance of synaptic plasticity are two linked, but independent processes. Upon induction of plasticity (LTP or LTD) a synaptic tag, which is protein synthesis-independent, is exhibit at activated synapses. The trigger for protein synthesis depends on the strength of the initial stimulation: the weak stimulus is not able to trigger protein synthesis and a transient form of plasticity is expressed; the strong stimulus is able to trigger the synthesis of proteins, leading to the expression of a maintained form of plasticity (Uwe Frey et al., 1988; Kelleher et al., 2004; Roger L. Redondo & Morris, 2011; Wang, Redondo, & Morris, 2010).

The STC hypothesis proposed an interaction between an input-specific synaptic tag and the capture of PRP. In fact, due to the cooperative sharing of PRPs, the induction of a maintained form of LTP in one set of synapses stabilize the transient form of LTP in another set of synapses. The PRPs synthetized upon a strong stimulation are allocated to the weakly stimulated synapse and the transient form of LTP is converted into a maintained one, a mechanism called synaptic cooperation (U. Frey & Morris, 1998; R. L. Redondo et al., 2010) **[Fig 1.1]**. The synaptic cooperation paradigm is based on two assumptions: (1) the weakly and the strongly stimulated synapses belong to two different axonal pools (2) the weakly and the strongly stimulated synapses belongs to the same post-synaptic neuron (Pinho,

Marcut, & Fonseca, 2020). Additionally, other aspects around this cooperative mechanism should be taken into consideration. The activation of the synaptic tag is transient, lasting about 30-60 min upon LTP induction. This means that the integration of the neuronal events, by cooperative maintenance, occurs within a specific time-window (U. Frey & Morris, 1998). One the other hand, the distance between the activated synapses is also an issue. Previous work from Govindarajan *et al*., demonstrate that synaptic capture efficacy decreases with increasing distance between synapses, and the synapses that are more than 70 μm apart are unable to cooperate (Govindarajan, Israely, Huang, & Tonegawa, 2011).

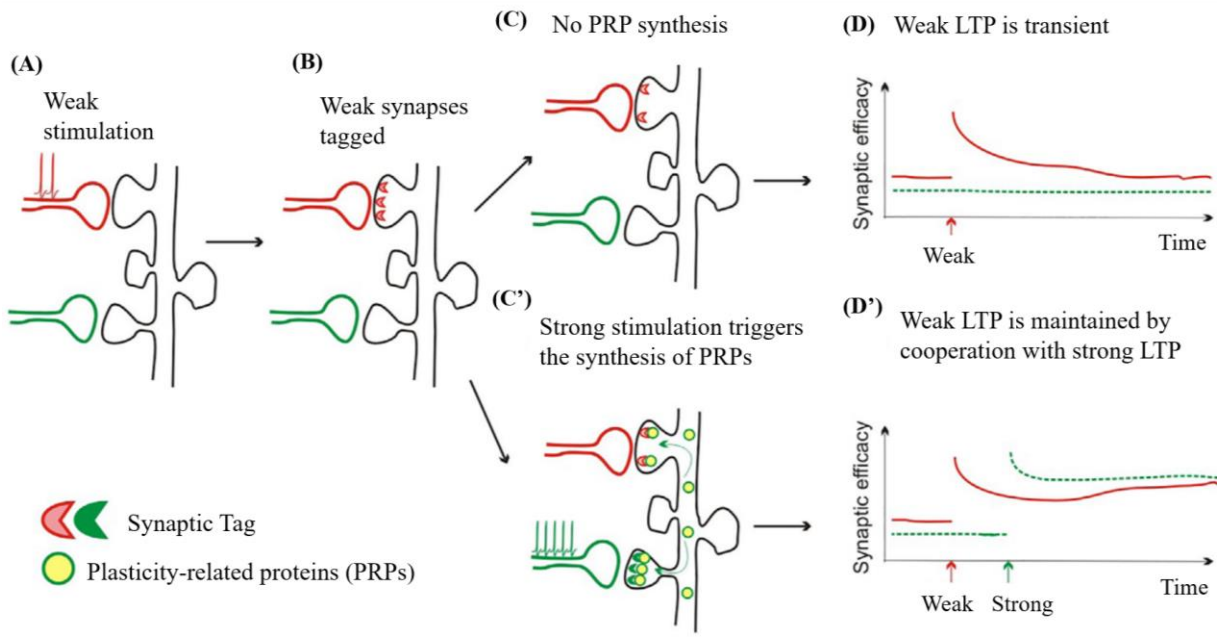


Figure 1.1 The synaptic tagging and capture hypothesis. **(A, B)** A weak stimulation of one pathway leads to the synaptic tag setting in an input-specific manner. **(C, D)** The stimulation of a synapse with a weak stimulus leads to the synaptic tag setting but not to the synthesis of PRPs, leading to a transient form of LTP that decays to the baseline value after a few hours. **(c', d')** The stimulation of a second set of synapses with a strong stimulus triggers PRPs synthesis allowing LTP maintenance. The weakly stimulated synapses that have access to the PRPs are also converted into a persistent LTP (cooperation mechanism). These PRPs are captured by the synapses that exhibit the tag, leading to the expression of maintained forms of LTP. PRP, plasticity-related proteins; LTP, long term-potentiation. Adapted from Pinho, Marcut & Fonseca, 2020, IUBMB Life.

The STC model showed for the first time that synapses are able to cooperate by sharing of proteins. Previous work of Fonseca *et.*, showed that synapses can also compete for the allocation of the PRPs to the tagged synapses. The authors showed that synapses engaged in a synaptic competition under a regime of low availability of PRPs or if the pool of the stimulated synapses are increased (Fonseca et al., 2004). Thus, the stimulated synapses can exhibit different patterns of plasticity depending on the number of tagged synapses and on PRPs availability **[Fig 1.2].**

Although STC hypothesis was proposed more than 20 years ago, there are still several questions regarding the molecular nature of the PRPs and the synaptic tag. Previous studies have suggested that PRPs are widely synthesized in the soma or dendrites upon a strong stimulation. Some of the proteins that have been proposed as PRPs include Homer a1 and brain-derived neurotrophic factor (BDNF) (Lanahan & Worley, 1998; Sajikumar & Korte, 2011; Sutton & Schuman, 2006).

The nature of the synaptic tag is also under intense investigations. Over the years, several molecules have been proposed for the synaptic tag role including CaMKII and actin cytoskeleton. However, previous studies showed that activation of CaMKII upon LTP induction seems to be increased

about 1-2 min, which is incompatible with the synaptic tag duration (Lee, Escobedo-lozoya, Szatmari, & Yasuda, 2009; Sanhueza & Lisman, 2013). The actin cytoskeleton has been proposed as a candidate for the synaptic tag due to its role in functional and structural plasticity. Expression of persistent forms of plasticity involves the remodeling of the actin cytoskeleton and pharmacological modulation of actin dynamics blocks the synaptic capture, suggesting a critical role of the actin in the STC hypothesis (Fonseca, 2012; C.-H. Kim & Lisman, 2018; Krucker, Siggins, & Halpain, 2000).

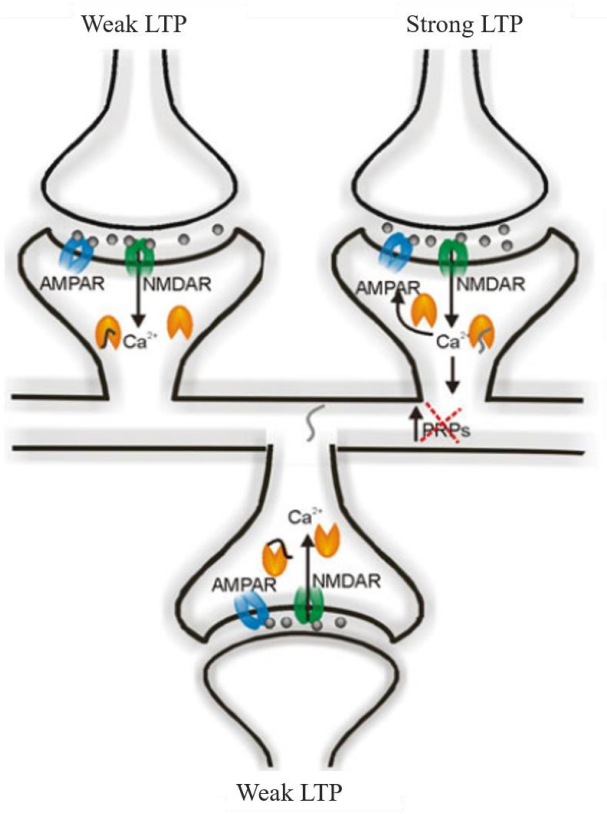


Figure 1.2 Synaptic competition. The stimulation of one of the synapses with a weak tetanic stimulation leads to a synaptic tag (yellow triangles) setting. A strong stimulation pathway triggers both PRPs synthesis and the synaptic tag setting. The activation of a third input with a weak stimulation leads to competition. LTP, long-term potentiation; PRPs, plasticity-related proteins; AMPA, α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; NMDA, N-methyl-D-aspartate. Adapted from Fonseca, 2015. Chapter in the book "Synaptic tagging and capture: From synapses to behavior.

1.3 Actin Cytoskeleton in Synaptic Plasticity

The STC hypothesis states that long-lasting forms of LTP are achieved based on the interplay between the synaptic tag and the capture of PRPs. Previous work in synaptic plasticity have suggested that any candidate for the synaptic tag role should fulfill the following criteria: 1) it should be activity-dependent; 2) it should be spatially restricted; 3) it should be able to interact with the PRPs in order to achieve the stabilization of LTP (K. C. Martin & Kosik, 2002). The actin cytoskeleton is a suitable candidate for the synaptic tag role.

The actin cytoskeleton is a complex structure within the cell and is one of the major components of the cellular scaffolding, being responsible for cell shaping, motility, and division (Carlier, 2010; Cingolani & Goda, 2008; Korobova & Svitkina, 2010). Within the cell, actin exists in two states: as a helical filament (F-actin) or as a monomer (G-actin), which provide the building blocks for F-actin assembly (Carlier, 2010; Cingolani & Goda, 2008; Korobova & Svitkina, 2010).

These two states of actin undergo a phenomenon known as “treadmilling”. The monomeric subunits (G-actin) with bound ATP are added to the fast-growing end (barbed end or plus end) and the monomeric subunits bound ADP are dissociated from the filaments at the other side (pointed end or minus end) (Honkura, Matsuzaki, Noguchi, Ellis-Davies, & Kasai, 2008; Nakahata & Yasuda, 2018).

In neurons, actin cytoskeleton plays a key role in axon and dendritic formation, contributing to neuron morphology. At the synaptic level, actin cytoskeleton is one of the major cytoskeletal proteins present in presynaptic terminals and postsynaptic dendritic spines. Previous studies have shown that F-actin can be divided into three different pools, based on its turnover rate. The pool of actin found at the tip and close to the postsynaptic density (PSD) of the dendritic spine presents a high turnover rate; the spine head presents an intermediate turnover pool and the spine neck exhibit a slow turnover pool of actin (Honkura et al., 2008; Pinho et al., 2020). Changes in synaptic strength observed after plasticity induction are associated with specific modification of dendritic spines morphology in an activity-dependent manner (Borczyk, Alicja, Caly, Bernas, & Radwanska, 2019; Borovac, Bosch, & Okamoto, 2018; Bosch & Hayashi, 2012; Lai & Ip, 2013) Induction of synaptic plasticity leads to a remodeling of the actin cytoskeleton, which in turn is associated with dendritic spine’s formation, elimination and morphology (Basu & Lamprecht, 2018; Dillon & Goda, 2005; Fukazawa et al., 2003; C.-H. Kim & Lisman, 2018).

The modulation of the actin cytoskeleton is also essential for the functional synaptic plasticity. Previous work showed that pharmacological modulation of actin cytoskeleton dynamics, through the application of drugs that affects its polymerization or depolymerization, interferes with the maintenance of LTP and synaptic capture, suggesting that a dynamic actin cytoskeleton is required for LTP maintenance (Borovac et al., 2018; Fonseca, 2012; Krucker et al., 2000; Ramachandran & Frey, 2009). The modulation of the actin cytoskeleton dynamics is a critical process in both structural and functional plasticity, and a tight control on actin cytoskeleton remodeling is required to proper synaptic function.

1.4 Regulation of Actin Dynamics

The actin cytoskeleton is essential for several functions in neurons and for that requires a dynamic interaction with different actin-binding proteins (ABP) such as Cofilin and CaMKII. These actin dynamics regulators are activated/inactivated in an activity-dependent manner, modulating the rate of actin assembly and disassembly during plasticity induction (Dos Remedios et al., 2003; Mitchison, Cramer, & Francisco, 1996; Winder & Ayscough, 2005). Actin depolymerizing proteins (ADF) of the ADF/Cofilin family modulate the actin cytoskeleton dynamics by inducing the severing of pre-existing actin filaments in a concentration-dependent manner. At low concentrations, cofilin promotes F-actin disassembly by accelerating the dissociation of monomeric actin (G-actin) from the filament minus ends, while at high concentrations cofilin promotes F-actin assembly by nucleating new and by stabilizing preexisting filament (Andrianantoandro & Pollard, 2006; Rust, 2015). The ADF/cofilin activity is regulated via phosphorylation at a serine residue at position 3 (Ser3) by LIM-kinases (LIMKs) and dephosphorylated by slingshot (SSH) family of protein phosphatases. Phosphorylation of cofilin leads to its inactivation and decreases its affinity to bind F-actin, promoting spine enlargement due to actin polymerization (Huang, DerMardirossian, & Bokoch, 2006; Meng et al., 2002; Takahashi et al., 2003).

CaMKII is one of the best-known ABP implicated in synaptic plasticity and modulation of actin dynamics (Fonseca, 2012; Khan, Conte, Carter, Bayer, & Molloy, 2016; Szabó, Manguinhas, & Fonseca, 2016; Szmek & Blackwell, 2019). This molecule can stabilize the actin cytoskeleton by crosslinking actin in bundles or linking actin to PSD proteins (Borovac et al., 2018; K. I. Okamoto,

Narayanan, Lee, Murata, & Hayashi, 2007). Once CaMKII is activated, several downstream signaling molecules including small GTPase proteins (RhoA, Rac1 and Cdc42) are activated at the stimulated spines (J. Li et al., 2016; Lisman, Yasuda, & Raghavachari, 2012; Murakoshi, Wang, & Yasuda, 2011) These small GTPases are highly present in the brain and act as an intracellular molecular switcher by changing their conformation between an active form (GTP-bound) and an inactive form (GDP-bound). They are bi-directionally regulated by GTP exchanger factor (GEF) and GTP accelerating protein (GAP). GEF facilitates the exchange of GTD for GTP, while GAP increases the endogenous GTPase activity of Rho GTPase (Luo, 2000). In their active state, Rho GTPase interacts with several downstream effectors modulating the actin cytoskeleton dynamics.

Upon plasticity induction, the entrance of calcium activates a complex molecular cascade that culminates with Cdc42, RhoA and Rac1 activation. Previous studies demonstrated that activation of Rac1 and Cdc42 promotes spine formation and enlargement by inducing F-actin polymerization. This requires activation of several downstream effectors such serine/threonine kinase p21-activated kinase (PAK) and LIM-kinase (LIMK), which ultimately inhibits cofilin activity (Spiering & Hodgson, 2011; Szmek & Blackwell, 2019). Additionally, Rac1 and Cdc42 can also promote actin polymerization through activation of WASP-family verprolin homologs (WAVE) and neural Wiskott Aldrich syndrome protein (N-WASP) respectively. Activated N-WASP and WAVE bind to actin-related proteins 2 and 3 complex (Arp2/3), stimulating the nucleation of actin (Y. Kim et al., 2006; Nakahata & Yasuda, 2018) [**FIG 1.3]**.

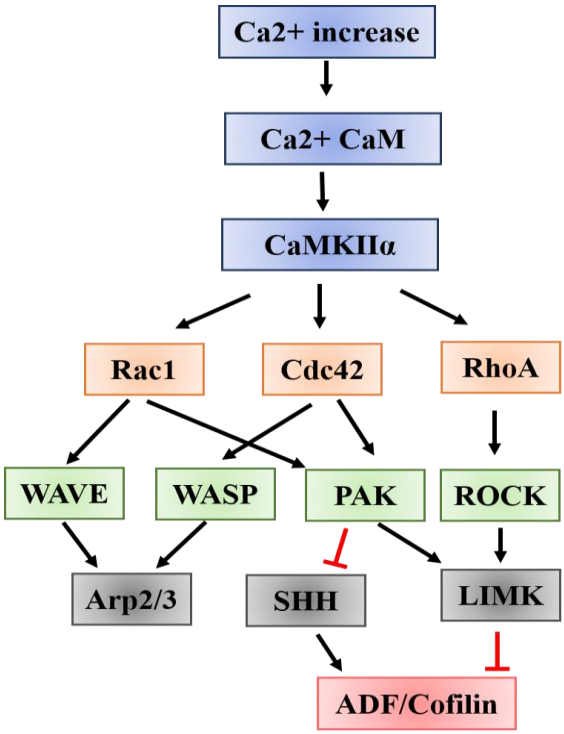


Figure 1.3 Rho GTPase and signaling networks. Plasticity induction leads to an increase of the calcium levels in the spine. Such elevation of calcium levels activates CaMKII, resulting in the subsequent activation of Rac1, Cdc42 and RhoA. Cdc42 and Rac1 activate the Wiskott-Aldrich Syndrome family of proteins including N-WASP and WAVE respectively, inducing polymerization of actin through Arp2/3 complex. Rac1 and Cdc42 also activate PAK1, which in turn activates LIMK. The phosphorylation of cofilin (inactive) prevents its actin-binding function, promoting actin polymerization. N-WASP, neuronal Wiskott-Aldrich syndrome protein; WAVE, WASP-family verprolin homologs; PAK, p21-activated kinase; LIMK, LIM-kinase; Arp2/3, actin-related proteins 2 and 3 complex. Adapted from Nakahata & Yasuda, 2018, Frontiers in Synaptic Neuroscience.

In 2011 Yasuda *et al.*, used two-photon fluorescence lifetime and two-photon glutamate uncaging to assess the activity of RhoA and Cdc42 upon synaptic plasticity induction. The authors

showed that the spatial role of these proteins is different: while RhoA spread out from the activate spines to the dendric shafts, Cdc42 is restricted to the activated spine (input-specific). Interestingly, the restricted activation of Cdc42 was not related with the limited diffusion of Cdc42 but rather with its inactivation immediately after diffusing out of the spines (Harvey, Yasuda, Zhong, & Svoboda, 2008; Murakoshi et al., 2011; Nakahata & Yasuda, 2018; Spence & Soderling, 2015). The role of Cdc42 activation in synaptic plasticity was assessed by Kim and colleagues in 2014. The authors showed that deletion of Cdc42 from the excitatory neurons leads to an impairment in synaptic plasticity, affecting both LTP maintenance and structural plasticity of the dendritic spines in the CA1 pyramidal neurons (I. H. Kim, Wang, Soderling, & Yasuda, 2014).

Taking into consideration the diffusion pattern of Cdc42 upon plasticity induction and its role in the actin dynamics, it seems relevant to address the role of Cdc42 activation in the modulation of the setting for the synaptic tag.

2. RATIONALE AND AIMS

The main goal of this work is to explore the role of the actin cytoskeleton dynamics as the synaptic tag of the potentiated synapses. Given that Cdc42 is an activity-dependent modulator of actin, that is spatially restricted to the stimulated spines, we suggest that activation of Cdc42 has a critical role in modulating of the synaptic tag.

In this context, the specific aims of the present work were:

1. Assess the role of Cdc42 activation in LTP induction and maintenance;
2. Assess the role of Cdc42 activation in synaptic cooperation;
3. Assess the role of Cdc42 activation in synaptic competition.

3. METHODS

3.1 Ethical statement

All procedures were approved by the Portuguese Veterinary Organization (DGAV) and are under the Decree-Law No. 113/2013 of 7 August (based on the EU Directive No. 2010/63 on the protection of animals used for scientific or educational purposes).

3.2 Animals

Wistar Han rats (21-35 days old) were bread at the housing facility of the host institution (CEDOC/FCM NOVA Medical School-Lisbon, Portugal). Rats were housed in plastic cages and maintained on a 14h light/10h dark cycle. Food and water were provided *at libitium*.

3.3 Slice preparation

All experiments were performed in transverse acute hippocampal slices. The animals were decapitated under isoflurane anesthesia and the brains were quickly removed and immersed in ice-cold *cutting* artificial cerebrospinal fluid (ACSF), saturated with 95% O_2/5% CO_2 and containing 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 5 mM $MgCl_2$, 1 mM $CaCl_2$ and 25 mM glucose The transverse hippocampal slices (400 μm thick) were obtained using a tissue slicer (Siskiyou MX-TS). Slices were maintained in a *cutting* ACSF at 32ºC for at least 1h before being transferred to the recording chamber for field electrophysiological recordings. The *recording* ACSF was saturated with 95% O_2/5%CO_2 and contained 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2 mM $MgCl_2$, 2.8 mM $CaCl_2$ and 25 mM glucose.

3.4 Electrophysiological recordings

Field excitatory postsynaptic potentials (fEPSP) were recorded in acute hippocampal slices placed in a recording chamber containing the *recording* ACSF at 32ºC. fEPSP, were recorded in the *stratum radiatum* of the CA1 region using a glass microelectrode filled with 3 M NaCl immobilized with 1% agarose (tip resistance of 1-3 MΩ). The stimulating electrodes (monopolar epoxy-insulated tungsten electrodes, Science Products, Germany) were placed on Schaffer collateral projecting from CA3 to CA1, positioned at an adequate distance from each other in order to stimulate two independent sets of Schaffer collaterals (S1 and S2) **[Fig 3.1]**. Stimulus intensity were set to evoke 50% of maximal fEPSP slope and LTP was induced after recording a stable baseline of fEPSP for 20 min. fEPSP were continuously recorded for 210 minutes. The test pulse frequency for each pathway was 0.033 Hz.

3.5 Induction of synaptic plasticity

Two stimulating electrodes (S1 and S2) were positing in the *stratum radiatum* layer, allowing the activation of two independent sets of Schaffer collaterals. To assess pathway independence, a paired-pulse facilitation (PPF) protocol was applied. Pathway independence was assessed by applying two pulses with 30 ms interpulse interval to the two pathways. The absence of crossed PPF was confirmed if no changes in the signal of any of the pathways were observed. After recording a stable baseline of fEPSP for 20 min, one of the pathways was arbitrarily selected to receive LTP-inducing stimulation.

The non-stimulated pathway was used as a control pathway. The stimulated pathway receives either weak tetanic stimulation (25 pulses at a frequency of 100 Hz, repeated 2 times, with an interval of 3 s), capable to induce transient forms of LTP or a strong tetanic stimulation (25 pulses at a frequency of 100Hz, repeated 5 times, with an interval of 3 s) capable to induce a persistent form of LTP. For experiments that require the activation of a third pathway (synaptic cooperation and competition experiments), a third stimulating electrode was used to stimulate an independent input in the antidromic direction (S3). In synaptic cooperation experiments (weak-before-strong protocol), a weak tetanic was induced in one pathway (S1) and 30 min later, the second pathway was stimulated with a strong tetanic stimulation (S2). The third electrode was used to assess slice viability (S3). In synaptic competition experiments, a weak tetanic stimulation was induced in one pathway (S1) and 30 min later, the second pathway was stimulated with a strong tetanic stimulation (S2). The third electrode was used to induce weak tetanic stimulation in a third pathway, 20 min after the strong stimulation (S3).

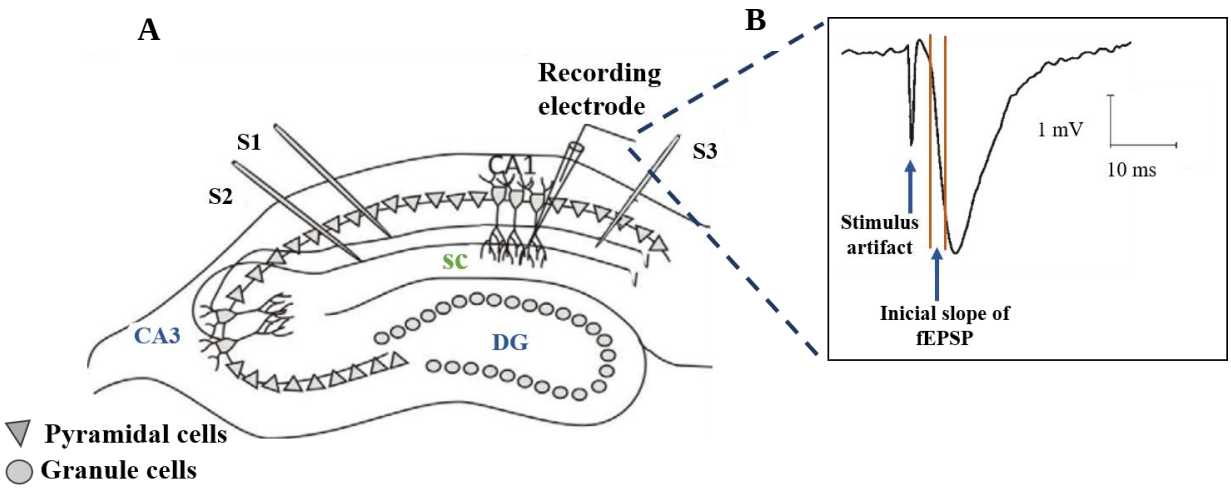


Figure 3.1 Schematic representation of a transversal hippocampal slice with the positioning of the electrodes for field-potential recordings (A) Two stimulating electrodes S1 and S2 were placed to activate two independent sets of Schaffer collaterals; a third stimulating electrode S3 was used for experiments that require the activation of three pathways. The recording electrode was placed on the *stratum radiatum* of the CA1 region to record fEPSP. **(B)** A representative fEPSP obtained by recording in the CA1 region in response to electrical stimulation of the Scaffer Collaterals. The first downward deflection is the stimulus artifact and the second is the synaptic response. The initial slope of a fEPSP is measured between the two orange lines. CA1-*cornu ammonis* region 1, CA3-*cornu ammonis* region 3, DG-dentate gyrus, SC-Shaffer collaterals, S1-stimulating electrode 1, S2-stimulating electrode 2, S3-stimulating electrode 3, fEPSP-field excitatory postsynaptic potentials. Adapted from Szabó *et al*., 2016, Nature.

3.6 Drug treatment

The following drugs were dissolved in (0.005%) Dimethyl sulfoxide (DMSO) and diluted to achieve the final concentration: ML141 (TargetMol) 5 µM, Cytochalasin B (Sigma) 0.5 µM. For the control experiments, only DMSO (0.005%) was added to the ACSF.

ML141 and Cytochalasin B act on actin cytoskeleton dynamics, modulating its polymerization. ML141 is a potent, selective and reversible non-competitive inhibitor of Cdc42, which blocks the binding of Cdc42 to guanosine triphosphate (inactive Cdc42), preventing actin polymerization (Hong et al., 2013; Surviladze, Waller, Strouse, & Bologa, 2010). Cytochalasin B is a cell-permeable mycotoxin which blocks G-monomers addiction to the barbed end of the actin filaments, preventing actin polymerization (MacLean & D.Pollard, 1980).

3.7 Experimental design

Initially, we stimulated one pathway with a weak tetanus and we bath-applied ML141 for 30 min, 10 min prior LTP induction (10 to 40 min). For the control experiments, only DMSO was bath-applied **[Fig 3.2]**.

Then, in a second set of experiments, we induce a persistent form of and ML141 was bath-applied at three different time points: 10 min prior LTP induction **(**10 to 40 min), 40 min upon LTP induction (60 to 90 min) and 70 min upon LTP induction (90 to 120 min). For the control experiments, only DMSO was bath-applied.

In the third set of experiments, we also addressed the role of Cdc42 inhibition during synaptic cooperation (weak-before-strong protocol). We bath-applied ML141 for 30 min between the weak and strong stimuli (20 to 50 min). The application of ML141 was also paired with Cytochalasin (ML141+ Cytochalasin) and with activity suspension (ML141+ NTP-No Test Pulse). For the control experiments, only DMSO was bath-applied.

In the last set of experiments, we addressed the role of Cdc42 activation in synaptic competition and we bath-applied ML141 for 30 min between the Strong and Weak2 stimuli (50 to 80 min). For the control experiments, only DMSO was bath-applied.

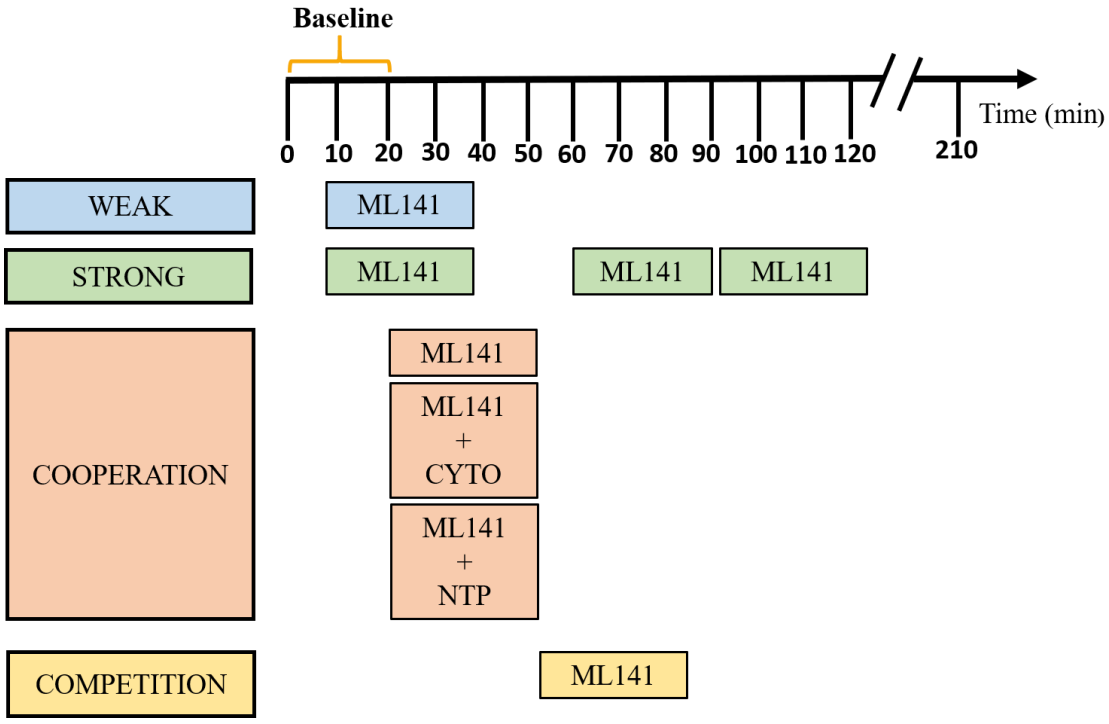


Figure 3.2 Experimental design. In experiments that only a weak stimulation was delivered, ML141 was bath-applied for 30 min, 10 min prior to LTP induction. The role of Cdc42 activation was also assessed upon a strong stimulation, and the ML141 was bath-applied for 30 min from 10-30 min, 60-90 min, and from 90-120 min. In the cooperation experiments, ML141 was bath-applied for 30 min (ML141). The bath-application of ML141 was also paired with the application of Cytochalasin (ML141+Cyto) and with the suspension of activity (ML141+NTP). In the competition experiments, ML141 was bath applied for 30 min, within the window from 50 to 80 min. NTP, No Test Pulse; Cyto, Cytochalasin B.

3.8 Data acquisition and analysis

Electrophysiological data were collected using a Dagan IX2-700 amplifier (Dagan, Minnesota, USA) filtered at 1 kHz using a LHBF 48X from NPI Electronic, GmbH, Germany. Data were sampled using a Lab-PCI-6014 (National Instruments, Austin, TX, USA) with a sampling rate of 10 kHz and stored on a computer. For offline data analysis, a customized LabView program was used (LabView 8.2.1, National Instruments, Austin, TX, USA).

As a measure of synaptic strength, the initial slope of the evoked fEPSP was calculated and expressed as percent changes from the baseline mean. Data were plotted using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). For the statistical analysis, the percentage of LTP decay was calculated by $(T_{initial}-T_{final})/T_{initial}$ x100, where $T_{initial}$ corresponds to the average of LTP values in the first 10 min after induction of LTP and the T_{final} corresponds to the averaged values of fEPSP slope between 190 to 200. All experiments in which fEPSP slope values of the control pathway decayed more than 20% were excluded. To test for group differences between LTP decay values across tested conditions, we confirmed normal distribution (Shapiro-Wilk test) and homoscedasticity (Levene test). Next, we performed a one-way ANOVA. For data sets that did not show a normal distribution, a non-parametric Kruskal-Wallis test was performed. For the correlation analysis, a Pearson analysis was performed and the percentage of LTP decay was calculated by $(T_{initial}-T_{final})/T_{initial}$ x100. All statistical analysis mentioned above were performed with a 0.05 significance level using IBM SPSS Statistics (IBM, North Castle, NY, USA.

4.1 *Preliminary Results*

To analyze the role of Cdc42 activation in the modulation of the synaptic tag, fEPSP were recorded from acute hippocampal slices upon inhibition of Cdc42, by ML141 bath-application, at different time points. Preliminary results demonstrated that induction of transient forms of LTP are not affected by the inhibition of Cdc42, while the induction of maintained forms of LTP are Cdc42 activation-dependent. The data also showed that Cdc42 activation is required within a certain time-window (20 to 90 min) upon induction of a maintained form of LTP. The preliminary experiments also showed that inhibition of Cdc42 blocks synaptic cooperation.

4.1.1 Maintained forms of LTP requires Cdc42 activation

The role of Cdc42 activation was investigated in the induction of transient forms of LTP. The results showed that inhibition of Cdc42 during 30 min (starting 10 min prior LTP induction) does not affect the induction of transient forms of LTP. Synaptic activation with a weak stimulation leads to a transient form of LTP that returns to the baseline values at the end of the recordings **[Fig 4.1]**. Analysis of LTP decay for the time window T1 (10 to 20 min) and T4 (190 to 200 min), showed no significant differences between the two conditions, ML141 22.86 ± 11.70, n=9 and DMSO 33.09 ± 6.54%, n=9 **[Fig 4.4A]**. The non-stimulated pathways served as control showing no impairment in synaptic transmission during 210 min of recording. These results indicate that the induction of transient forms of LTP, due to a weak tetanic stimulation, are not affected by the Cdc42 inhibition.

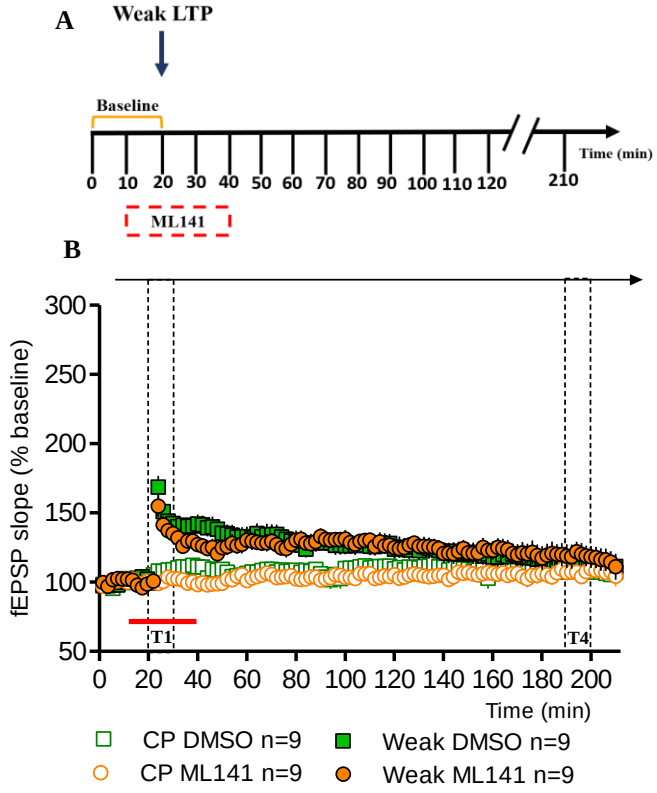


Figure 4.1 Inhibition of Cdc42 does not affect the induction of transient form of LTP. (A) Diagram showing the induction of a weak LTP at 20 min with ML141 bath-application from 10 to 40 minutes. **(B)** Bath-application of ML141 for 30 min does not affect the transient form of LTP (●) For the control experiments, only DMSO (0.005 %) was applied (■). Slice viability was

assessed by recording a second independent pathway that did not show a significant decrease throughout the recording (□○). The red line represents the time-window of the drug application. Data are represented as mean ± SEM. N=number of slices, CP-control pathway, SEM-standard error of the mean. T1=20 to 30 min; T4=190 to 200 min. Data reproduced from Nunes, M. *Master Thesis*, 2019.

The role of Cdc42 activation in the induction of a maintained form of LTP, using a strong tetanic stimulation, was also tested. Interestingly, the results revealed that bath-application of ML141 during 30 min (starting 10 min prior to LTP induction) affects the induction of a maintained form of LTP. The inhibition of Cdc42 leads to an unstable form of LTP which returns to the baseline values at the ends of recordings [**Fig 4.2**]. Analysis of LTP decay for the time window T1 (10 to 20 min) and T4 (190 to 200 min) showed a significant increase in LTP decay in the ML141-treated slices (44.28 ± 11.09, n=8) when compared with DMSO-treated slices (15.35 ± 5.59, n=10) [**Fig 4.4B**]. These results suggest that the induction of maintained forms of LTP are Cdc42 activation-dependent.

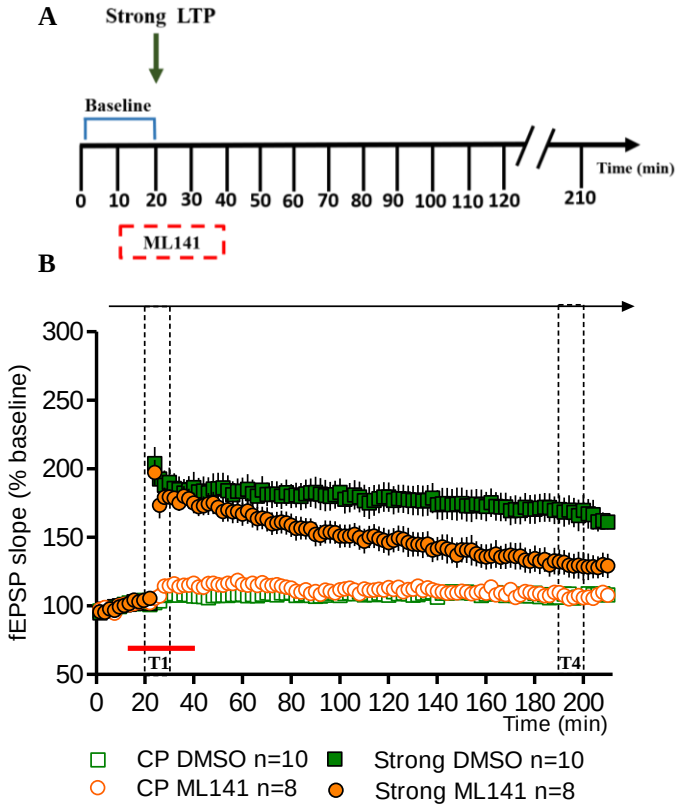


Figure 4.2 Induction of maintained forms of LTP are blocked by Cdc42 inhibition. (A) Diagram showing the induction of a strong LTP at 20 min with ML141 bath-application from 10 to 40 minutes. **(B)** Bath-application of ML141 for 30 min affects the induction of a maintained form of LTP (●). For the control experiments, only DMSO (0.005 %) was applied (■). Slice viability was assessed by recording a second independent pathway that did not show a significant decrease throughout the recording (○□). The red line represents the time-window of drug application.Data are represented as mean ± SEM. N=number of slices, CP-control pathway, SEM-standard error of the mean. T1=20 to 30 min; T4=190 to 200 min. Data reproduced from Nunes, M. *Master Thesis*, 2019.

Given that the previous results indicated that inhibition of Cdc42 at the time of LTP induction affects the expression of the maintained forms, the role of Cdc42 activation upon the induction of the maintained form of LTP was also investigated. The results showed that inhibition of Cdc42 between 60 to 90 minutes (40 min upon LTP induction) destabilized the maintained forms of LTP **[Fig 4.3A].** The non-stimulated pathways served as a control pathway showing no impairment in synaptic transmission during 210 min of recording. Analysis of LTP decay for the time window T2 (50 to 60 min) and T4 (190

to 200 min), demonstrated a significant increase in the LTP decay in the ML141-treated slices (25.10 ± 5.49, n=9) when compared with DMSO-treated slices (5.83 ± 2.94 n=10) [**Fig 4.4C**].

Moreover, the role of Cdc42 activation was also tested between 90 to 120 minutes. The results showed that bath-application of Cdc42 inhibitor does not interfere with LTP maintenance when applied 70 min upon LTP induction **[Fig 4.3B].** Analysis of LTP decay for the time window T3 (80 to 90 min) and T4 (190 to 200), showed no significant difference in the percentage of LTP decay between the ML141-treated slices (7.40 ± 3.89 n=8) and DMSO-treated slices (12.32 ± 1.90 n=8) [**Fig 4.4D**]. This data suggested that Cdc42 plays an important role in LTP maintenance within a certain time-window.

Together, these data showed an important role of Cdc42 activation in the induction and expression of long-lasting forms of plasticity, through modulation of the actin cytoskeleton, in a time-dependent manner.

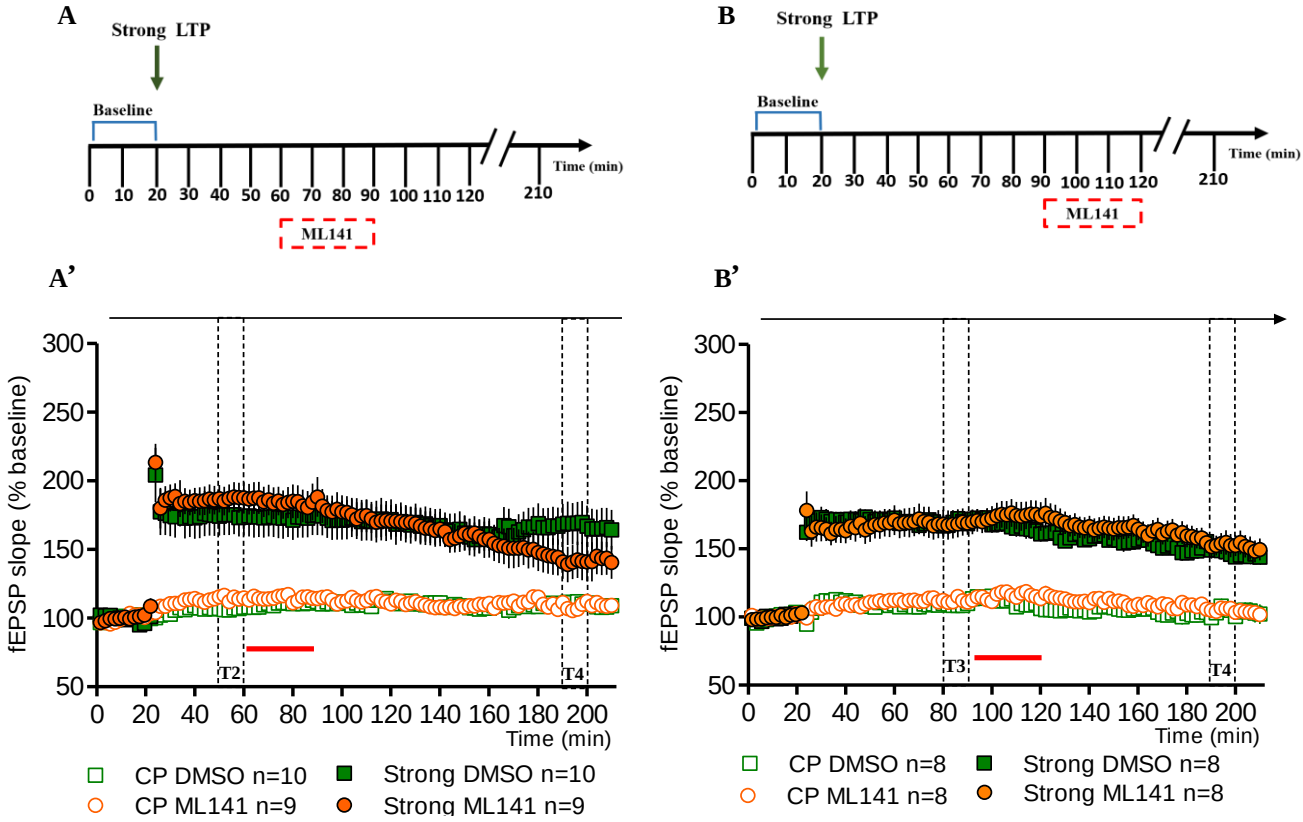


Figure 4.3 Inhibition of Cd42 destabilizes LTP maintenance in a time window dependent-manner. (A) Diagram showing the induction of a strong LTP at 20 min with ML141 application from 60 to 90 minutes. **(A')** Bath-application of ML141 for 30 min affects the maintenance of a persistent form of LTP (●). For the control experiments, only DMSO (0.005 %) was applied (■) Slice viability was assessed by recording a second independent pathway that did not show a significant decrease throughout the recording (○□) Data are represented as mean ± SEM. **(B)** Diagram showing the induction of a strong LTP at 20 min with ML141 bath-application from 90 to 120 minutes. **(B')** Induction of LTP with a strong tetanic stimulation leads to a maintained form of LTP. Bath-application of ML141 for 30 min does not affect the maintained form of LTP (●). For the control experiments, only DMSO (0.005 %) was applied (■).Slice viability was assessed by recording a second independent pathway that did not show a significant decrease throughout the recording (○□). The red line represents the time-window of drug application. Data are represented as mean ± SEM. N=number of slices, CP-control pathway, SEM-standard error of the mean. T2=50 to 60 min; T3=80 to 90 min; T4=190 to 200 min. Data reproduced from Nunes, M. *Master Thesis*, 2019.

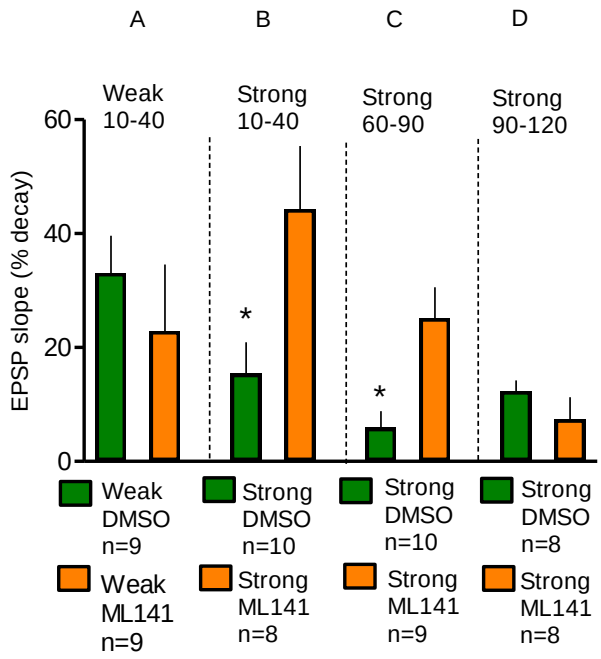


Figure 4.4 Summary plot showing the percentage of LTP decay between tested conditions. (A) Inhibition of Cdc42 for the time window 10 to 40 min does not show significant differences in LTP decay between ML141-treated slices (▭) and DMSO-treated slices (▭) (A one-way ANOVA test was performed F (1.16) = 0.59; P = 0.46). The percentage of LTP decay was calculated between T1 (10 to 20 min) and T4 (190 to 200 min). **(B)** Inhibition of Cdc42 for the time window 10 to 40 min show an LTP decay significantly higher in ML141-treated slices (▭) compared to DMSO-treated slice (▭) (A one-way ANOVA test was performed F (1.16)= 6.13; P = 0.002). Percentage of LTP decay was calculated between T1 (10 to 20 min) and T4 (190 to 200 min) **(C)** Inhibition of Cdc42 for the time window 60 to 90 min show an LTP decay significantly higher in ML141-treated slices (▭) compared to DMSO-treated slice (▭) (A one-way ANOVA was performed F (1.17) = 15.42; P = 0.001. The percentage of LTP decay was calculated between T2 (50 to 60 min) and T4 (190 to 200 min) **(D)** Inhibition of Cdc42 for the time window 90 to 120 min does no show a significant difference in LTP decay between ML141-treated slices (▭) and DMSO-treated slices (▭) (A one-way ANOVA test was performed F (1.14) = 1.15; P = 0.30). The percentage of LTP decay was calculated between T3 (80 to 90 min) and T4 (190 to 200 min). Data reproduced from Nunes, M. *Master Thesis*, 2019.

4.1.2 Inhibition of Cdc42 blocks synaptic capture

In order to test the role of Cdc42 activation in synaptic cooperation, by synaptic tagging mechanism, a weak-before-strong protocol was performed. The results showed that the stimulation of a second independent pathway with a strong tetanus, 30 min after the weak stimulus, is able to convert the transient form of LTP into a maintained form of LTP. A third stimulation electrode was also used to assess slice viability, showing no differences throughout the recordings [**Fig 4.5A].**

Next, the same weak-before-strong protocol as before was used and the inhibition of Cdc42 activation occur between the weak and the strong stimuli. The results showed that bath-application of ML141 blocks synaptic cooperation. The behavior of a control pathway was also assessed, and no difference was observed during the recordings.

In another set of experiments, one pathway was stimulated with a weak tetanus (Weak alone), and the behavior of this pathway was recorded together with the behavior of two unstimulated pathways. The data show that the stimulated pathway exhibit a transient form of LTP that returns to baseline values **[Fig 4.5B]**.

Analysis of LTP decay for the time window T2 (50 to 60 min) and T4 (190 to 200 min) showed a significant increase in the percentage of LTP decay between the ML141-treated slices (ML141 19.00 ± 3.65) and the control (DMSO) (controls -2.70 ± 2.19, n=9) and also a significant increase of LTP

decay in the "weak alone" (weak alone 17.35 ± 10.47 n=7) and the control (DMSO) (controls -2.70 ± 2.19, n=9) **[Fig 4.8]**. These results suggest that the maintenance of LTP, by the synaptic capture mechanism, is Cdc42 activation-dependent.

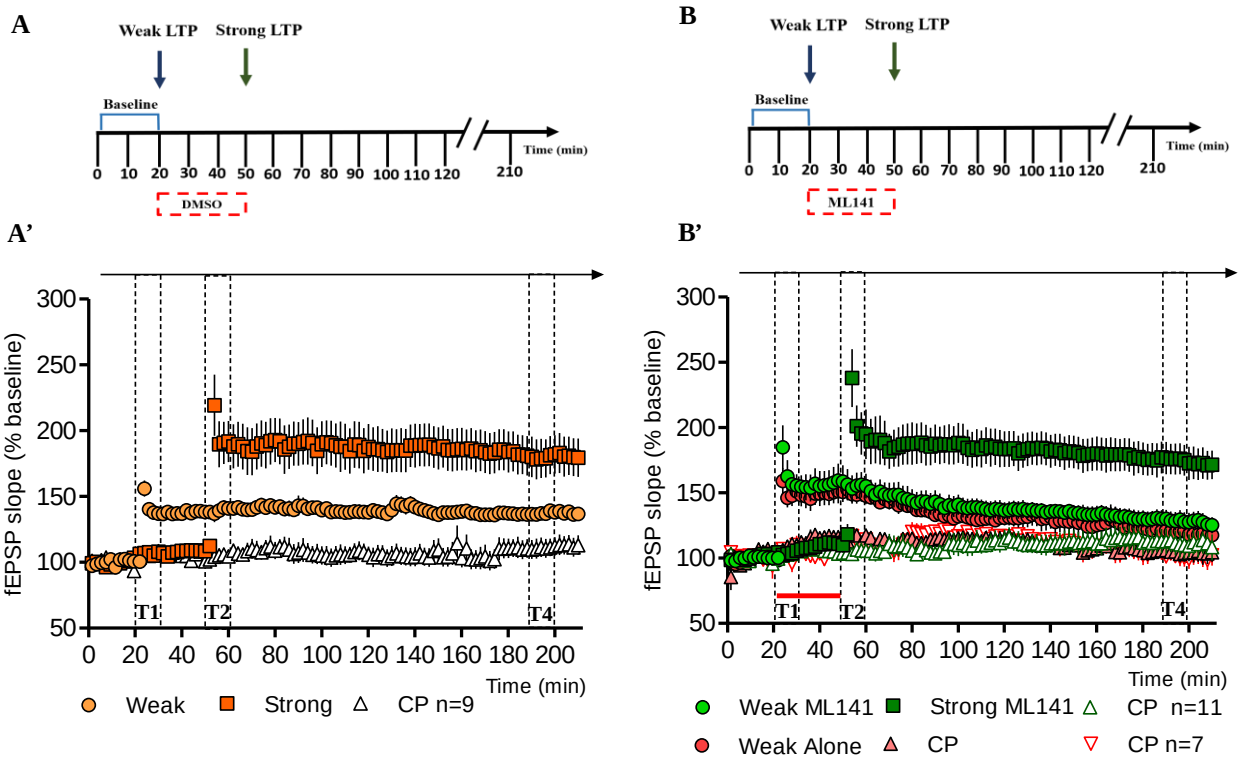


Figure 4.5 Inhibition of Cdc42 activation blocks synaptic capture. (A) Timing scheme for the synaptic competition experiments with bath-application of DMSO. **(A')** A weak tetanic stimulation induced at 20 min (○) is converted into a maintained form of LTP due to the induction of strong tetanic stimulation (■) 30-min later. DMSO (0.005 %) was added to the ACSF for 30 min between the weak and the strong stimuli (20 to 50 min). No changes were observed in the slice viability (△). Data are represented as mean ± SEM**. (B)** Timing scheme for the synaptic competition experiments with bath-application of ML141. **(B')** Application of ML141 for 30 min between the weak tetanic stimulation (○) and the strong tetanic stimulation (■) blocks the conversion of the transient form of LTP into a persistent form. A control pathway was also recorded to assess slice viability (△). In the second set of experiments, one pathway was stimulated with a weak tetanus was at 20-min (○), while the second (▽) and the third (△) unstimulated pathways were also recorded. The red line represents the time-window of drug application T4. Data are represented as mean ± SEM**.** N=number of slices, CP-control pathway, SEM-standard error of the mean. T1=20 to 30 min; T=50 to 60 min; T4=190 to 200 min. Data reproduced from Nunes, M. *Master Thesis*, 2019

4.2 Results

Taking into consideration the preliminary results from our lab, where it was clearly showed that Cdc42 activation play an important role in synaptic capture mechanism, we continue to address the role of Cdc42 in synaptic cooperation but also in synaptic competition. Our results demonstrated that the synaptic capture blockade, due to ML141 bath-application, can be reverted when an actin polymerization inhibitor was present or when synaptic activity was suspended. Also, we showed that inhibition of Cdc42 blocks the synaptic competition mechanism.

4.2.1 Inhibition of actin polymerization and suspension of activity restores synaptic capture

The effect on synaptic capture observed in the presence of ML141 was similar to that observed in our previous work when Jasplakinolide, an actin depolymerization inhibitor, was present. Bath-application of Jasplakinolide for 30 min between the weak and the strong stimuli blocks the stabilization of the transient form of LTP (Fonseca, 2012). Given the impairment on synaptic capture under the inhibition of Cdc42, a molecule involved in polymerization of actin, we use Cytochalasin, a well-known actin polymerization inhibitor, and we test its effect on synaptic cooperation.

We analyzed the effect of simultaneous application of ML141 and Cytochalasin in the synaptic capture phenomenon. Our results reveal that the co-application of ML141 and Cytochalasin for 30 min between the two stimuli restores the impairment in the synaptic capture observed when only ML141 was applied **[Fig 4.6]**. Analysis of LTP decay for the time window T2 (50 to 60 min) and T4 (190 to 200 min) showed a significant increase in the percentage of LTP decay between the ML141-treated slices (ML141 19.00 ± 3.65) and the ML141+Cytochalasin-treated slices (ML141+Cyto 1.73 ± 6.00 n=1) **[Fig 4.8]**. These results suggested that actin dynamics is required for the synaptic capture mechanism and its pharmacological modulation is able to restore the synaptic tag.

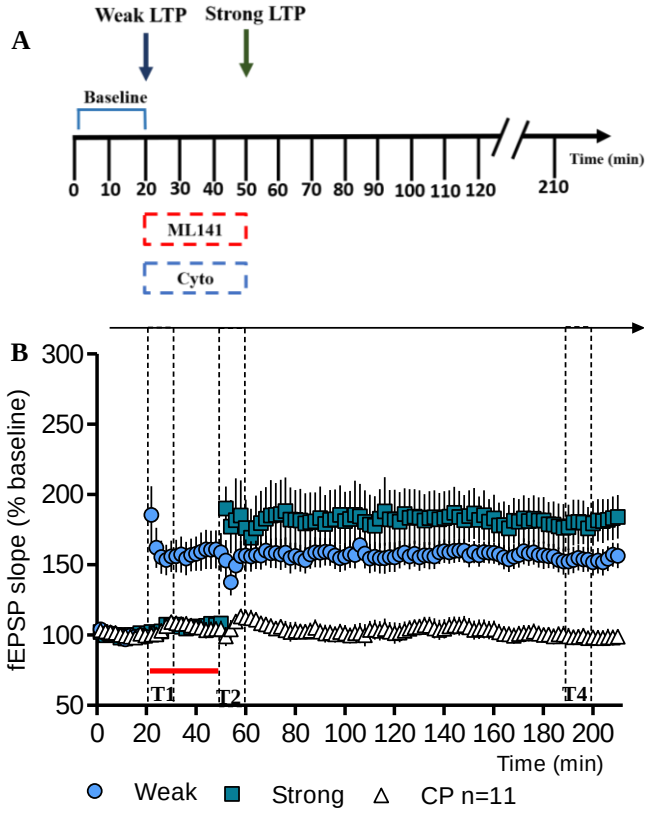


Figure 4.6 The application of cytochalasin restores the synaptic capture blockade induced by Cdc42 inhibition. (A) Timing scheme for cooperation LTP experiments with co-application of ML141 and cytochalasin from 20 to 50 minutes. **(B)** Co-application of ML41 and Cytochalasin, between the weak (○) and strong stimuli (■), restores the synaptic capture of the weak tetanic stimulated pathway. A control pathway was also recorded to assess slice viability (△). The red line represents the time-window of drug application. Data are represented as mean ± SEM. n=number of slices, CP-control pathways, SEM-standard error of the mean. T1=20 to 30 min; T2=50 to 60 min T4=190 to 200 min.

Previous work from our lab showed that the suspension of the test pulse stimulation (NTP-no test pulse) during the time of cytochalasin application was sufficient to rescue the impairment of LTD maintenance induced by the drug (Szabó et al., 2016). Given this, we tested whether suspension of activity during Cdc42 inhibition affects synaptic cooperation. Using the same cooperation approach as before, we paired ML141 bath-application with activity suspension (ML141+NTP). Our results showed that suspension of the test pulse stimulation during the time of ML141 application restores synaptic cooperation. The behavior of a control pathway was also assessed, and no difference was observed during the recordings **[Fig 4.7]**.

Analysis of LTP decay for the time window T2 (50 to 60 min) and T4 (190 to 200 min) showed a significant increase in the percentage of LTP decay between the ML141-treated slices (ML141 19.00 ± 3.65) and the ML141+ NTP-treated slices (ML141+NTP 1.90 ± 6.67) **[Fig 4.8]**. We showed that when the test pulse stimulation was suspended, the administration of ML141 did not affect synaptic cooperation, suggesting that the effect of Cdc42 inhibition in synaptic cooperation is highly related with actin cytoskeleton modulation and with on-going activity.

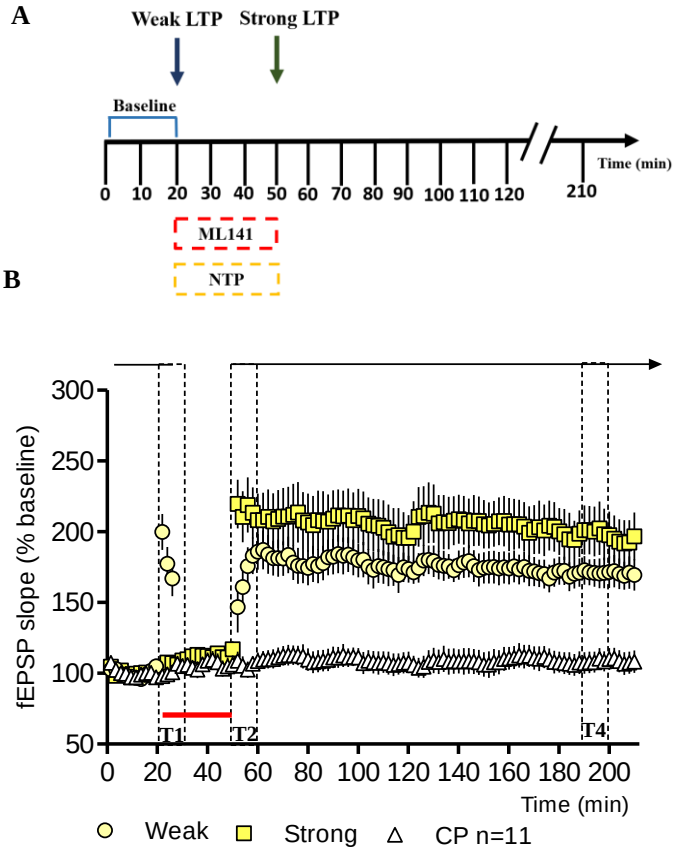


Figure 4.7 Suspension of activity restores the synaptic cooperation (A) Timing scheme for cooperation LTP experiments with ML141 application and suspended activity from 20 to 50 minutes. **(B)** Suspension of test pulse stimulation between the weak (○) and the strong stimulation (□) during ML141 application is sufficient to rescue the synaptic capture blockade. A control pathway was also recorded to assess slice viability (△). The red line represents the time-window of drug application.

Data are represented as mean ± SEM**.** n=number of slices, CP-control pathway, NTP-No test-pulse, SEM-standard error of the mean. T1=20 to 30 min; T2=50 to 60 min T4=190 to 200 min.

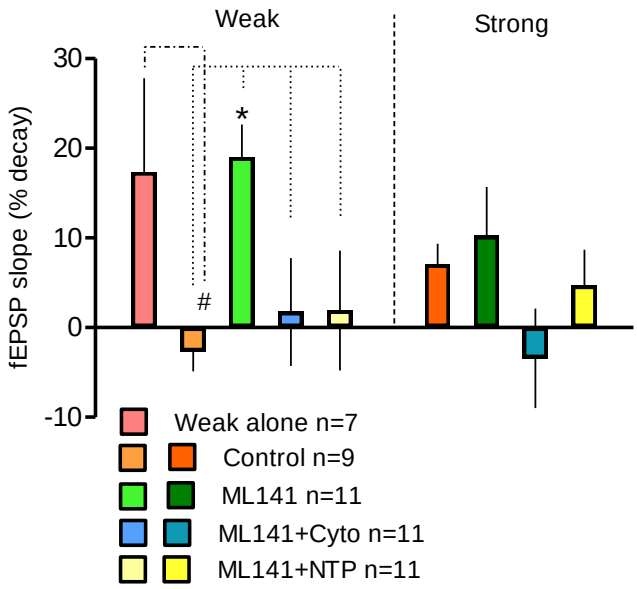


Figure 4.8 Summary plot showing the percentage of LTP decay between the tested conditions. Inhibition of Cdc42 between the weak and the strong stimulation (20 to 50 min) blocks the synaptic capture mechanism. The ML141-treated slices show an LTP decay significantly higher than ML141+Cyto and ML141+NTP (ANOVA test was performed F (4.46) = 3.02; P = 0.027). n=number of slices.

4.2.2 Cdc42 inhibition affects synaptic competition

The role of Cdc42 activation was also assessed in the synaptic competition using a weak-strong-weak protocol. In our competition protocol, the stimulation of one pathway with a strong tetanic stimulation induces the synthesis of PRPs that are shared between three stimulated pathways. This protocol enables us to increase the pool of activated synapses, while the amount of PRPs available are shared among those tags. First, we showed that the activation of a third pathway is able to induce synaptic competition **[Fig 4.9A]**.

Next, we inhibit Cdc42 activation during 30 min between the Strong and the Weak2 stimulation (50-80 min) and we observed that bath-application of ML141 abolish the ability of the pathways to compete. Interestingly, we showed that inhibition of Cdc42 upon the strong stimulation allows the maintenance of all pathways **[Fig 4.9B]**.

We conducted a correlation analysis during the administration of DMSO, and we plotted the percentage of potentiation of one pathway against the percentage of LTP decay of the next stimulated pathway. The percentage potentiation of Weak1 and Strong pathways positively correlates with the percentage of LTP decay the Strong the Weak2 pathways, respectively. As the percentage of LTP potentiation increases, the percentage of LTP decay of the next stimulated pathway also increases, which may suggest that the capture of PRPs by one pathway reduces the pool of proteins available for the next stimulated pathway to capture **[Fig 4.10]**.

Analysis of LTP decay for the time window T3 (70 to 80 min) and T4 (190 to 200 min) revealed a significant increase in the percentage of LTP decay between the Weak2 DMSO-treated slices (Weak2 DMSO 36.11 ± 3.62 n=10) and the Weak2 ML141-treated slices (Weak 2ML141 8.31 ± 5.46, n=10) **[Fig 4.11]**. Together, our results showed that inhibition of Cdc42 leads to the maintenance of all activated inputs, even under a regime of reduced PRPs availability.

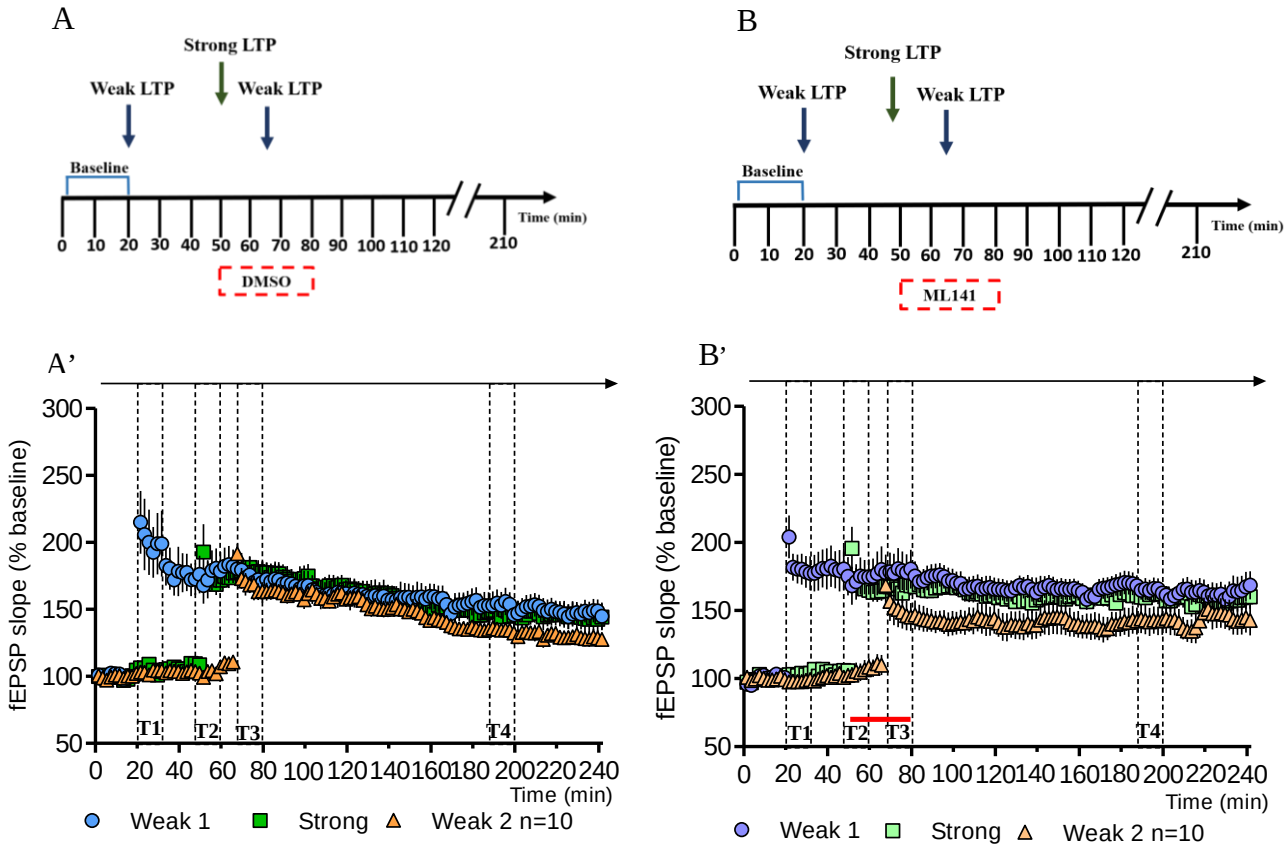
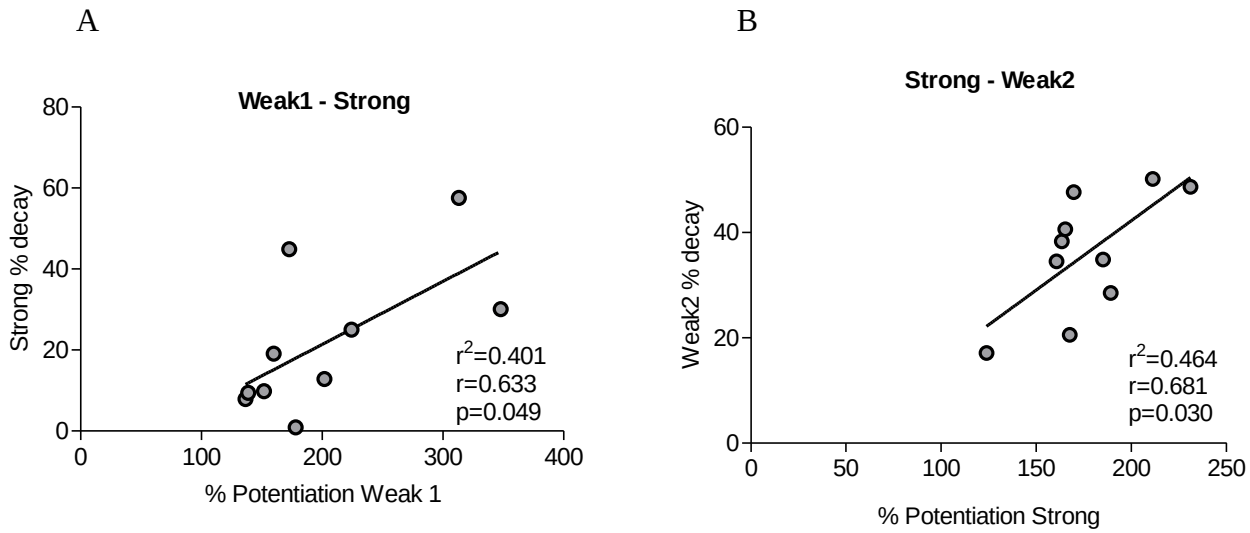


Figure 4.9 Inhibition of Cdc42 interferes with synaptic competition. (A) Timing scheme for competition LTP experiments with DMSO application between the strong and the weak2. **(A')** Stimulation of a pathway with a weak tetanus at 20 min (Weak1 ●) followed by a strong stimulation 30 min later (Strong ■), and a weak tetanus 15 min later (Weak2 ▲) induces synaptic competition. Data are represented as mean ± SEM. **(B)** Timing scheme for competition LTP experiments with ML141 application between the strong and the weak2. **(B')** Bath application of ML141 between the Strong (□) and Weak2 (△) interferes with the synaptic competition. The red line represents the time-window of drug application. Data are represented as mean ± SEM. n=number of slices, CP-control pathway, SEM-standard error of the mean. T1=20 to 30 min; T2=50 to 60 min; T3=70 to 80 min; T4=190 to 200 min



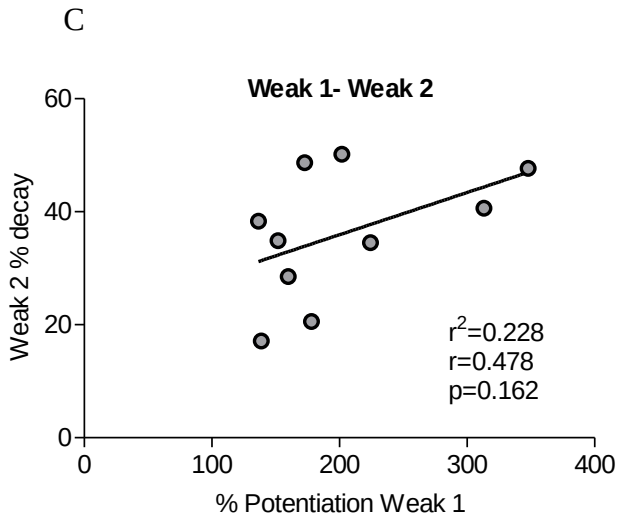


Figure 4.10 The percentage of potentiation of one pathway correlates with the percentage of LTP decay of the next stimulated pathway. (A) Correlation plot of the percentage of potentiation of the Weak1 against the percentage decay of the Strong pathway. The significant positive correlation showed that a higher percentage of Weak1 potentiation increases the percentage decay of the Strong. **(B)** Correlation plot of the percentage of potentiation of the Strong against the percentage decay of the Weak2 pathway. The significant positive correlation showed that a higher percentage of Strong potentiation increases the percentage decay of the Weak2. **(C)** Correlation plot of the potentiation of the Weak1 against the percentage decay of the Weak2 pathway. The correlation coefficient is not statistically significant (p=0.162).

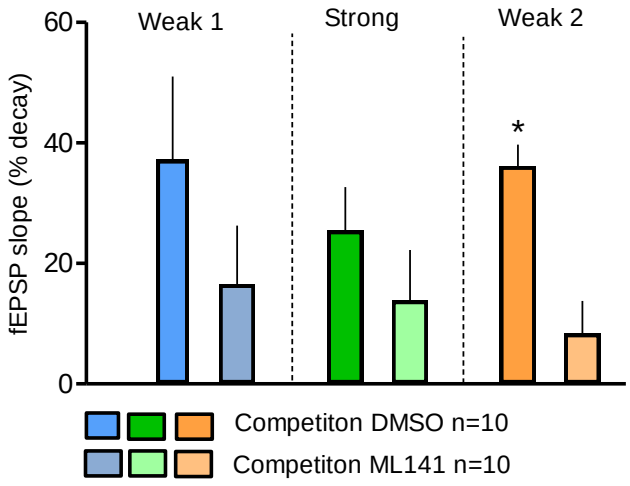


Figure 4.11 Summary plot showing the percentage of LTP decay between tested conditions. Inhibition of Cdc42 for 30 min between Strong and Weak 2 blocks synaptic competition. The percentage of LTP decay between Weak1 ML141 and Weak1 Control (A one-way ANOVA test was performed (1.18) = 1.48; P = 0.24. The percentage of LTP decay between Strong ML141 and Strong Control (ANOVA with a Fisher post hoc test was performed (1.18) = 1.1; P = 0.31. The percentage of LTP decay between Weak2 ML141 and Weak2 Control (ANOVA test was performed (1.18) = 18.024; P < 0.001.

5. DISCUSSION AND CONCLUSION

The STC hypothesis proposes that the maintenance of long-lasting forms of plasticity depends on the interplay between an input-specific synaptic tag and PRPs. The generated synaptic tag should be able to capture the PRPs, which in turn are needed for the stabilization of the potentiated state (Uwe Frey & Morris, 1998; R. L. Redondo et al., 2010). Several studies have been conducted in order understand the molecular identity of the synaptic tag and many of them have suggested actin cytoskeleton as a major candidate for the synaptic tag (Fonseca, 2012; K. C. Martin & Kosik, 2002; K. Okamoto, Bosch, & Hayashi, 2009; Szabó et al., 2016). In this study, we addressed the role of Cdc42 activation in the modulation of the synaptic tag. Upon LTP induction, Cdc42 is a spatially restricted molecule to the stimulated synapses (input-specific) and an activity-dependent modulator of the actin cytoskeleton dynamics (Murakoshi et al., 2011; Nakahata & Yasuda, 2018). Given this, we assume that interfering with molecules that are involved in actin dynamics, the setting for the synaptic tag will be also affected. Here, we showed that induction of transient forms of LTP are not affected by inhibition of Cdc42, while the induction of maintained forms of LTP are blocked. Additionally, we also showed that the maintained forms of LTP can be destabilized if the Cdc42 inhibition occurs until one hour upon LTP induction. Our data also demonstrated that inhibition of Cdc42 interferes with both synaptic cooperation and competition.

According to STC hypothesis, induction of LTP with a weak tetanic stimulation leads to a transient form of LTP that decays to the baseline values after a few hours. We found that inhibition of Cdc42 activation for 30 min (10 min prior LTP induction) does not present an effect on the induction of a transient form of LTP. This is consistent with the idea that LTP maintenance is dependent on PRPs synthesis and their capture by the synaptic tag. In the absence of a strong stimulation, the synthesis of PRPs is not triggered and stabilization of the weakly stimulated pathway does not occur (U. Frey & Morris, 1998). We also found that bath-application of ML141 for 30 min (10 min prior LTP induction) blocks the induction of a maintained form of LTP. Previous studies have suggested that maintained forms of LTP require the capture of PRPs by the tag exhibit at the activated synapses. Although the strong stimulation leads to PRPs synthesis, the inhibition of Cdc42 alters the actin cytoskeleton dynamics, and the interaction between synaptic tag-PRPs is compromised. These results support the hypothesis that a dynamic actin cytoskeleton is required at the time of induction for LTP maintenance, and inhibition of such dynamics interferes with the stabilization of the activated inputs (Fukazawa et al., 2003; Krucker et al., 2000). Additionally, we found that inhibition of Cdc42 activation is able to destabilize the maintained forms of LTP if ML141 bath-application occurs within 70 min upon LTP induction, while inhibition of Cdc42 70 min after LTP induction has no impact in LTP maintenance. These results suggest that Cdc42 activation is required for LTP maintenance in a time-window dependent manner. Our results are in agreement with previous data that showed that the time duration of the synaptic tag is about 1 hour (U. Frey & Morris, 1998; Sajikumar & Frey, 2004). Then, inhibition of Cdc42 70 min after induction does not interfere with LTP maintenance since the synaptic tag is no longer available.

Previous work from our lab showed that actin cytoskeleton modulation is also needed for the synaptic cooperation mechanism (Fonseca, 2012; Szabó et al., 2016). Using a synaptic tagging and capture experimental approach (cooperation protocol), we showed that inhibition of Cdc42 between the weak and the strong stimuli blocks the stabilization of the transient form of LTP by the synaptic capture mechanism. Previous studies from our lab showed that inhibition of actin depolymerization, through bath-application of Jasplakinolide, blocked synaptic cooperation (Fonseca, 2012). According to previous studies, the downstream effect of Cdc42 on the actin cytoskeleton is to promote its polymerization (Heasman & Ridley, 2008; Sit & Manser, 2011). Thus, it seems that both actin polymerization and actin depolymerization inhibition renders an similar effect on synaptic capture,

suggesting that both drugs block the stabilization of the transient's forms of LTP. This is consistent with the idea that interfering with actin cytoskeleton leads to an instability synaptic tag, which in turn affects the synaptic capture, suggesting that the integrity of the actin cytoskeleton is required for the synaptic cooperation.

Taking into consideration the effect of Jasplakinolide and ML141 on synaptic capture, we pair the bath-application of ML with Cytochalasin, a well-known actin polymerization inhibitor. Interestingly, we found that the synaptic capture blockade induced by ML141 bath-application can be restored with the simultaneous application of ML141 and Cytochalasin. These results present some incongruities since both ML141 and Cytochalasin bath-application should modulate the actin cytoskeleton in the same direction. One possible explanation for this observation is based on the presence of three different pools of actin at the spines (Honkura et al., 2008). It is possible that depending on the drug applied, ML141 or Cytochalasin, the pools of actin may exhibit an differentiated response regarding their modulation, which in turn will interfere with the plasticity outcome. Another possibility is that ML141 and Cytochalasin modulated actin cytoskeleton through different action mechanisms. While Cytochalasin inhibits the addition of G-monomers to the barbed end of the actin filaments, preventing actin polymerization, ML141 blocks the binding of Cdc42 to guanosine triphosphate, leading to an inactive Cdc42 (GDP-bound) (Hong et al., 2013; MacLean & D.Pollard, 1980). The inhibition of actin polymerization through Cdc42 inhibition open the possibility to other molecules involved in the singling pathway to interfere with the actin modulation. We also found that pairing ML141 bath-application with the suspension of the test-pulse stimulation (NTP) was able to restore the synaptic capture. These results are in line with previous studies that showed that the synaptic tag is activity-dependent (Fonseca, 2012; Szabó et al., 2016). Again, these results shows that a dynamics actin is needed to the maintenance of the long-lasting forms of plasticity, supporting the hypothesis of the actin cytoskeleton modulation as the synaptic tag.

Because the STC hypothesis is based on an interplay between the synaptic tag and PRPs, if the protein availability is limited, for example by increasing the pool of the activated synapses or by inhibiting PRPs synthesis, a synaptic competition mechanism is observed. Here, using a weak-strong-weak protocol, we increase the pool of activated synapses to which PRP needs to be allocated in order to stabilize the transient forms of LTP. Activation of multiple inputs generates a competitive pressure, since the PRPs would be distributed among all activated synapses, leading to a competition mechanism among the activated inputs for expression of a maintained form of LTP (Fonseca, 2015; Fonseca et al., 2004; Govindarajan et al., 2011). We showed that the activation of a third input induces synaptic competition. Based on the analysis of the correlation plots, we found that changes in the percentage of potentiation of one pathway are correlated with changes in the percentage decay of the next stimulated pathway, suggesting that the previously activated synapses are able to interfere with the maintenance of the future events. The influence of the prior events on the future events can be explain by the metaplasticity phenomenon. Metaplasticity was first described by Abraham and Bear in 1996 and states that prior activity events (priming event) are able to modulate the following response of the synapse, influencing the ability of the synapses to express plasticity. In fact, heterosynaptic metaplasticity proposed that synaptic activity at one synapse is able to interfere with the plasticity expression at the neighboring synapse (Abraham, 2008; Abraham & Bear, 1996; Q. Li et al., 2012; Sajikumar, Li, Abraham, & Xiao, 2009). Interestingly, we also found that under ML141 application synaptic competition is blocked, and all stimulated pathways are maintained. In fact, our result suggest that inhibition of Cdc42 between the Strong and the Weak2 lead to a cooperative phenomenon, where the two weakly stimulated synapses cooperate with the strong, leading to the stabilization of all pathways. These results are not consistent with previous knowledge regarding the impact of Cdc42 inhibition on synaptic tag. One possible explanation for this observation is that activation of three pathways together

with Cdc42 inhibition induces modifications in the neuronal basal threshold, changing the modulation of the plasticity events.

Taken together, our data present strong evidence the LTP maintenance involves the remodeling of the actin cytoskeleton, through activation of Cdc42, supporting the hypothesis of actin cytoskeleton as a synaptic tag. Induction of plasticity leads to a re-organization of the actin cytoskeleton, that changes the structural state of the synapse and provides the molecular signal for the input-specific capture of PRPs.

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